Received: 18 August 2019 | Revised: 27 October 2019 | Accepted: 15 November 2019

DOI: 10.1002/ardp.201900241

DPhG Arch Pharm
Archiv der Pharmazie

FULL PAPER

Design, synthesis, antileishmanial, and antifungal biological evaluation of novel 3,5-disubstituted isoxazole compounds based on 5-nitrofuran scaffolds

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Funding information
Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul, Grant/Award Numbers: 23/200.462/2014, 23/200.708/2013, 59/300.109/2015; Conselho Nacional de Desenvolvimento Científico e Tecnológico; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Abstract

Nineteen 3,5-disubstituted-isoxazole analogs were synthesized based on nitrofuran scaffolds, by a [3 + 2] cycloaddition reaction between terminal acetylenes and 5-nitrofuran chloro-oxime. The compounds were obtained in moderate to very good yields (45–91%). The antileishmanial activity was assayed against the promastigote and amastigote forms of *Leishmania (Leishmania) amazonensis*. Alkylchlorinated compounds **14p–r** were active on both the promastigote and amastigote forms, with emphasis on compound **14p**, which showed strong activity against the amastigote form (IC_{50} = 0.6 μM and selectivity index [SI] = 5.2). In the alkyl series, compound **14o** stands out with an IC_{50} = 8.5 μM and SI = 8.0 on the amastigote form. In the aromatic series, the most active compounds were those containing electron-donor groups, such as trimethoxy isoxazole **14g** (IC_{50} = 1.2 μM and SI = 20.2); compound **14h**, with IC_{50} = 7.0 μM and SI = 6.1; and compound **14j** containing the 4-SCH_3 group, with IC_{50} = 5.7 μM and SI = 10.2. In addition, the antifungal activity of 19 nitrofuran isoxazoles was evaluated against five strains of *Candida* (*C. albicans*, *C. parapsilosis*, *C. krusei*, *C. tropicalis*, and *C. glabrata*). Eleven isoxazole derivatives were active against *C. parapsilosis*, and compound **14o** was found to be the most active (minimal inhibitory concentration [MIC] = 3.4 μM) for this strain. Compound **14p** was active against all the strains tested, with an MIC = 17.5 μM for *C. glabrata*, lower than that of the fluconazole used as the reference drug.

KEYWORDS
5-nitrofuran scaffolds, antifungal activity, antileishmanial activity, drug design, isoxazole core

*Ozildéia S. Trefzger, Natália V. Barbosa, and Renata L. Scapolatempo contributed equally to this work.

1 | INTRODUCTION

Leishmaniasis is a neglected disease that affects mainly underdeveloped tropical countries located in Africa, Asia, and Central and South America. About one billion people around the world are at the risk of contracting this disease, with a high mortality rate, if not adequately treated. The main clinical manifestations of the disease are cutaneous, mucocutaneous, and visceral.[1]

The current treatment is limited to pentavalent antimonials, such as sodium stibogluconate, liposomal amphotericin B, miltefosine, pentamidine, and paromomycin. The available treatment is not optimal due to adverse effects, difficulty in use, parasite resistance, safety, and price.[2–4] Considering the socioeconomic conditions of the countries involved and increasing problems of resistance, programs to develop new, accessible drugs for leishmaniasis are imperative.

Candidiasis is a fungal infection caused by the *Candida* genus with diverse clinical manifestations, which include invasive candidiasis, a bloodstream infection that ranges from symptomatic candidaemia to fulminant sepsis.[5] The risk factors are long-term hospitalization of patients in the intensive care unit and use of central venous catheters and immunosuppressive agents.[6] Although *Candida albicans* is often the main cause of candidiasis, a shift has been observed from this species to nonalbicans *Candida*, mainly *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*.[7]

There are four classes of antifungal agents used in the treatment of candidiasis: azoles, echinocandins, polyenes, and pyrimidine analogues. However, they have several disadvantages in terms of toxicity, pharmacokinetic profile, and resistant strains.[8] For instance, *C. glabrata* shows the highest proportion of isolated strains resistant to azoles, such as fluconazole.[9]

Our research group studied molecular modification of tetrahydrofuran neolignans[10] like veraguensin **1**, grandisin **2**, and machilin G **3** with a potential antileishmanial activity using bioisosterism, as a tool for molecular modification, to produce new derivatives by replacing the tetrahydrofuran ring by an isoxazole core[11,12] and others[13,14] (Figure 1).

Isoxazoles are considered privileged scaffolds in drug discovery and have a broad spectrum of activities—including antibacterial,[15] antiviral,[16] anti-inflammatory,[17] antimycobacterial[18]—and more recently, they have also demonstrated antitrypanosomal activity.[19]

This nucleus is present in antifungal compounds, such as micafungin **7**[20] and azoles derivatives **8** (Figure 2).[21] The presence of the isoxazole nucleus increased the potency of micafungin **7** ten times compared to the natural echinocandin.[20] The azole derivative with isoxazole nucleus **8** showed activity (minimal inhibitory concentration [MIC] = 2 μg/ml) against fluconazole and voriconazole-resistant *C. glabrata* strains.[21]

On the other hand, the family of nitroheterocycles is relevant in the treatment against neglected diseases and also as antibacterial agents. In this family, it is important to highlight the compounds based on the 5-nitrofurans structures, such as nitrofurazone **9** and its hydroxylated derivative **10**, with potent antichagasic activity; nifurtimox **11**, a drug used to treat Chagas disease; and nifuroxazide **12**, a nitrofuran with potent antimicrobial and antiprotozoal activity (Figure 3).[22] Recently, Kamal et al.[23] synthesized a 5-nitrofuran–triazole congener **13** with antifungal properties, extending the antimicrobial activities of this scaffold.

Despite the applicability of nitroheterocyclic derivatives as antimicrobial and antiprotozoal agents, they have been the subject of intense discussions about their mutagenicity and carcinogenicity due to the bioreduction process of the nitro group.[24] However, compound **10** (NFOH-121) showed lower mutagenicity in the Ames test than nitrofurazone **9**,[22] and its nongenotoxicity was confirmed by micronucleus assay.[24]

Other drug discovery programs based on molecular modifications of nitroheterocyclic drugs resulted in the design of safe compounds for the treatment of neglected diseases. In this scenario, fexinidazole, an antitrypanosomal candidate drug, has been shown to be highly safe in clinical trials in humans performed by DNDi.[25,26]

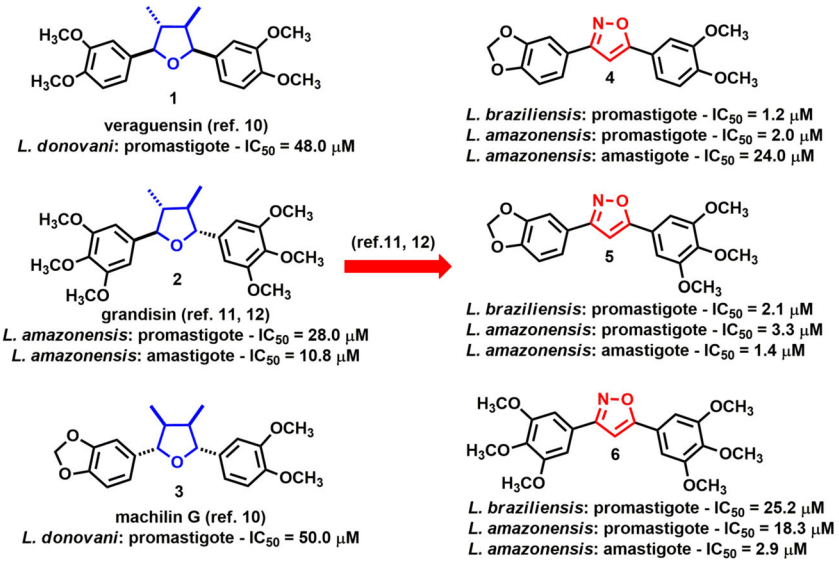


FIGURE 1 Antileishmanial activities of synthetic isoxazole **4–6** compounds based on neolignans **1–3**

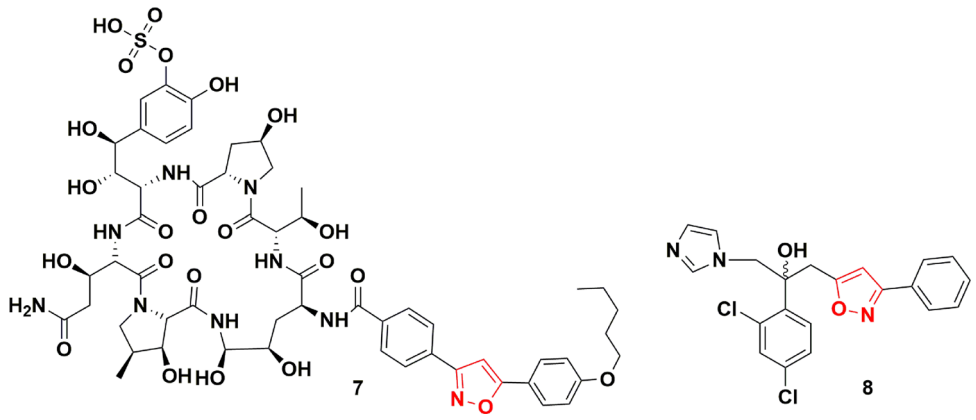
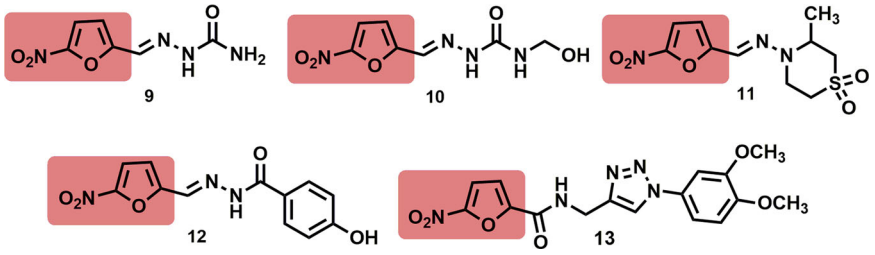


FIGURE 2 Antifungal isoxazole compounds micafungin **7** and azole derivative **8**

FIGURE 3 Structures of the bioactive nitrofuran compounds **9–13**



Among the 5-nitrofurans that are used as antiparasitic agents, those with a structural profile based on semicarbazones, hydrazones, acyl hydrazones, and modifications have been highlighted.[27]

To broaden the chemical space of the compounds obtained with this scaffold, this study aims to prepare a series of compounds using the isoxazole core as a linker between the 5-nitrofuran scaffold and various substituents R_1, and perform assays to explore their antileishmanial and antifungal activities (Figure 4).

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

Compounds were synthesized through a [3 + 2] cycloaddition reaction between terminal acetylenes with 5-nitrofuraldehyde chloro-oxime. The 5-nitrofuraldehyde chloro-oxime **17** was synthesized from 5-nitrofuraldehyde **15** in two steps (Scheme 1).[28]

For the synthesis of aryl acetylenes, first, compounds with aromatic substituents were prepared. Aryl acetylenes containing withdrawing and donor electron groups were synthesized by the Sonogashira cross-coupling reaction between 2-methyl-3-butyn-2-ol and aryl bromides or iodides using $Pd(PPh_3)_2Cl_2$, CuI in the presence of Et_3N as the reaction base (Scheme 2, procedure a).[11–14] Subsequently, the compounds were submitted to retro-Favorski reaction with KOH and the terminal acetylenes **22b–i** were obtained in good yields (Scheme 2, procedure b).[11–14] Phenylacetylene was obtained as a commercially available reagent. Ethynyl-1,2,3-trimethoxybenzene **22i** was synthesized by the Corey–Fuchs procedure (Scheme 2, procedures c and d).[11–14] The compound **25** was prepared through Sonogashira cross-coupling reaction between aryl iodide **23** and ethynyltrimethylsilane **24** (Scheme 2, procedure e).[11–14]

Cycloaddition [3 + 2] reaction was performed between 5-nitrofuran chloro-oxime **17** and terminal acetylenes **22a–i,** and trimethylsilylacetylene **25**, using a catalytic system of $CuSO_4{\cdot}5H_2O$, sodium ascorbate, and $KHCO_3$, in a solvent mixture of THF/CH_2Cl_2 1:1 providing nitrofuran isoxazoles **14a–j** in good yields (Table 1).[29]

The synthesis of alkylated and chloroalkylated substituted 3,5-isoxazoles **14k–r** (Table 1), and **14t** (Scheme 3) aimed to expand the proposed series to obtain more information about the structure–activity relationship (SAR). Alkyl- and chloroalkyl-substituted acetylenes **22k–q** and **22s** were obtained as commercially available reagents.

Compound **14t** was synthesized in two reaction steps from the [3 + 2] cycloaddition reaction of chloro-oxime **17** with 3-butyn-1-ol **22r**, and subsequent reaction of **14s** with cyanuric chloride (Scheme 3).[30]

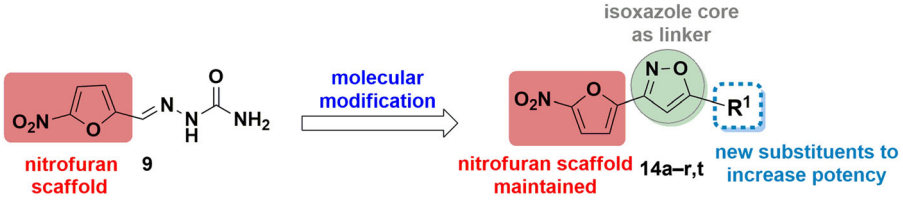
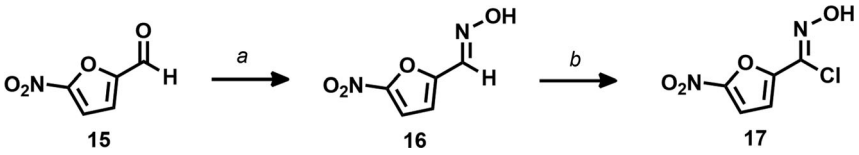


FIGURE 4 Design of novel 3,5-isoxazole nitrofuran analogues



SCHEME 1 Synthesis of 5-nitrofuraldehyde chloro-oxime **17**. Reagents and conditions: (a) $NH_2OH.HCl$, NaOH, H_2O/EtOH (2:3), r.t., 3 hr (yield = 80%); (b) NCS, DMF, 60°C, 2 hr (yield = 78%)

All synthesized compounds **14a–t** were identified by ^1H NMR, ^{13}C NMR, and high-resolution mass spectra (HRMS).

2.2 | Biology

2.2.1 | Antileishmanial activity

The aromatic compounds were first evaluated for antileishmanial activity on the promastigote forms of *Leishmania (Leishmania) amazonensis*. The initial objective was to screen 3,5-diaryl-isoxazoles containing electron donors and withdrawing groups to make a comparison with compound **14a**, and thus to understand the role of these groups in the possible antileishmanial activity (Table 2).

Compound **14a** and its chlorinated derivative **14b** were active against promastigotes of *L. amazonensis* (IC_{50} = 7.9 μM and 7.0 μM, respectively) and showed moderate activity against amastigote forms with an IC_{50} = 34.0 μM and 23.0 μM, respectively. Compound **14c** was inactive against both forms (IC_{50} > 150 μM).

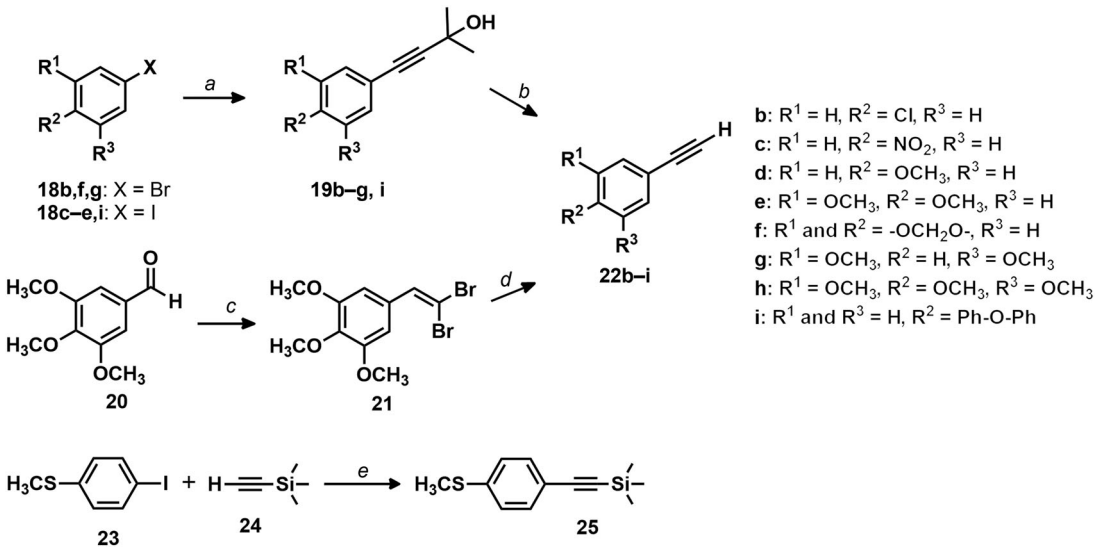
The methoxylated-substituted derivatives **14d–g** had interesting profiles of antileishmanial activity. Although the 4-methoxy and 3, 4-methoxyl substituted compounds **14d–e** had relevant activity against the promastigote forms (IC_{50} = 6.0 μM and 7.8 μM, respectively), compared to pentamidine (IC_{50} = 8.9 μM). The 3,5- and 3,4, 5-methoxylated-substituted derivatives **14f–g** were considered inactive. The activity profile changed when the series was evaluated against the amastigote forms.

The 3,4,5-methoxylated compound **14g** was the most active (IC_{50} = 1.2 μM and SI = 20.2) against amastigote forms and its 3,5-methoxy congener **14f** showed an IC_{50} of 19.5 μM.

Compound **14h**, containing the methylenedioxy group, showed activity against promastigotes and amastigotes with IC_{50} = 16.4 μM and 7.0 μM, respectively (Table 2). Compound **14i** (R_1 = Ph–O–Ph, Table 2) was more active against promastigote (IC_{50} = 1.7 μM) than against amastigote forms (IC_{50} = 16.6 μM). Compound **14j**, containing the 4-SCH_3 group, a more apolar bioisoster of the compound **14d** (containing the 4-OCH_3 group), had an IC_{50} of 5.7 μM against the amastigote forms of *L. amazonensis*.

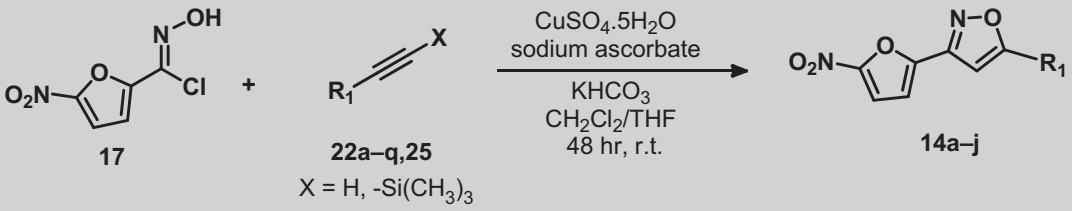
To expand the number of examples, alkylated homologous series **14k–o** were tested, in which the carbon chain number ranged from four to eight carbons.

SAR information reported in the literature supports that the homologation approach can be adopted in the discovery of new drug candidates.[31] The size of the carbon chain, in this case, affects directly the lipophilicity parameter (Table 3). In compounds **14k–n**, there is a tendency to increase antileishmanial activity over promastigote forms when ClogP increases to the limit of 5 (Table 4). However, the antileishmanial activity on amastigote forms showed an



SCHEME 2 Preparation of aryl acetylenes **22b–i** and **25**. Reagents and conditions: (a) 2-methyl-3-butyn-2-ol, $PdCl_2(PPh_3)_2$/CuI, Et_3N, reflux, 24 hr (yield: 55–93%); (b) KOH, toluene, reflux, 24 hr (yield: 43–90%); (c) CBr_4, PPh_3, CH_2Cl_2, 0°C, 5 hr (yield: 74%); (d) THF, *n*-BuLi, −25°C to room temperature, 1 hr (**22h**, yield: 83%). (e) $PdCl_2(PPh_3)_2$/CuI, Et_3N, reflux, 24 hr (yield: 73%)

TABLE 1 Synthesized 3,5-nitrofuran isoxazoles **14a–r** and **14t**



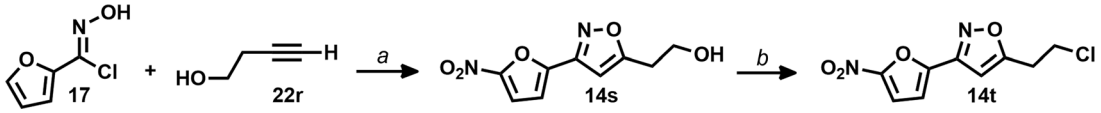
Compounds 14a–r	R_1	Yield (%)
14a	Ph	89
14b	4-Cl–Ph	55
14c	4-NO_2–Ph	45
14d	4-OCH_3–Ph	83
14e	3,4-*di*-OCH_3–Ph	85
14f	3,5-*di*-OCH_3–Ph	72
14g	3,4,5-*tri*-OCH_3–Ph	81
14h	3,4-methylenedioxy–Ph	74
14i	Ph–O–Ph	64
14j	4-SCH_3–Ph	58
14k	–C_4H_9	86
14l	–C_5H_{11}	70
14m	–C_6H_{13}	91
14n	–C_7H_{15}	89
14o	–C_8H_{17}	84
14p	–CH_2Cl	68
14q	–$(CH_2)_3Cl$	74
14r	–$(CH_2)_4Cl$	78

[a]Chlorooxime 17 (2.0 mmol), terminal acetylenes **22a–r**, **25** (2.0 mmol), $CuSO_4{\cdot}5H_2O$ (0.30 mmol), sodium ascorbate (0.50 mmol), $KHCO_3$ (20 mmol), THF (5 ml), CH_2Cl_2 (5 ml), r.t., 48 hr.

IC_{50} with a zig-zag tendency, when the biological activity alternates with the number of carbons present in the alkyl chain.[31] In this series, compound **14o** showed a relevant antileishmanial activity against promastigote and amastigote forms (IC_{50} = 6.1 μM and 8.5 μM, respectively).

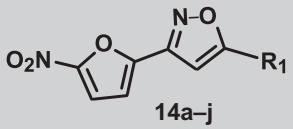
Inspired by the positive results found in the alkylated series (**14k–o**; Table 3), a homologous chlorinated alkyl series **14p–r** and **14t** was designed and tested to complement the SAR information. The number of –CH_2 groups ranged from one to four, and antileishmanial activity assay showed compounds with a good profile on both the promastigotes and amastigotes forms. Upon analysis of the antipromastigote activity, it was found that compounds **14o–r** showed a tendency of higher potency associated with increasing –CH_2 groups in the homologous series. Compound **14t** was an exception with poor activity toward promastigotes (Table 3). However, in the specific case of antileishmanial activity against amastigote forms, compound **14p** was the most active, with only one –CH_2 group (IC_{50} = 0.6 μM, SI = 5.2).

Compounds **14q**, **14r**, and **14t** had an IC_{50} between 3.2 μM and 4.1 μM, which is similar to the activity of the reference drug pentamidine (IC_{50} = 3.3 μM) on the amastigote forms of *L. amazonensis*, although the selectivity indexes (SI) were not satisfactory with values close to 1.



SCHEME 3 Synthesis of nitrofuran isoxazole derivative **14t**. Reagents and conditions: (a) $CuSO_4{\cdot}H_2O$, sodium ascorbate, $KHCO_3$, THF/CH_2Cl_2 (1:1), r.t., 48 hr (yield: 50%); (b) cyanuric chloride, DMF, CH_2Cl_2, r.t., 4 hr (yield: 65%)

TABLE 2 Antileishmanial activities of 3,5-diaryl-isoxazoles **14a–j**



14a–j

Compound/R_1	ClogP[a]	NIH/3T3 IC_{50} (μM) ± *SD*[b]	Promastigote IC_{50} (μM) ± *SD*[c]	SI[d]	Amastigote IC_{50} (μM) ± *SD*[e]	SI[f]
14a: R_1 = Ph	3.2	28.5 ± 0.2	7.9 ± 0.9	3.6	34.0 ± 1.5	0.8
14b: R_1 = 4-Cl–Ph	3.9	250 ± 2.3	7.0 ± 0.8	35.3	23.0 ± 1.3	10.8
14c: R_1 = 4-NO_2–Ph	3.2	NC	166 ± 2.2	NC	155 ± 1.2	NC
14d: R_1 = 4-OCH_3–Ph	3.3	25.4 ± 4.9	6.0 ± 0.7	25.4	31.8 ± 1.5	0.8
14e: R_1 = 3,4-*di*-OCH_3–Ph	2.9	36.4 ± 4.3	7.8 ± 0.8	4.7	14.6 ± 1.1	2.5
14f: R_1 = 3,5-*di*-OCH_3–Ph	3.3	250 ± 2.3	158 ± 2.2	2.6	19.5 ± 1.3	12.8
14g: R_1 = 3,4,5-*tri*-OCH_3–Ph	2.9	25.0 ± 2.1	144 ± 2.1	0.2	1.2 ± 0.0	20.2
14h: R_1 = 3,4-methylenedioxy–Ph	3.1	43.3 ± 6.0	16.4 ± 1.2	2.6	7.0 ± 0.8	6.1
14i: R_1 = Ph–O–Ph	5.0	250 ± 2.3	1.7 ± 0.2	142.0	16.6 ± 1.2	15.2
14j: R_1 = 4-SCH_3–Ph	3.7	57.9 ± 8.5	16.7 ± 1.2	3.5	5.7 ± 0.5	10.2
Doxorubicin[g]	–	0.7 ± 0.0	–	–	–	–
Pentamidine[g]	–	27.5 ± 3.1	8.9 ± 0.6	3.0	3.3 ± 0.3	8.3
Amphotericin B	–	2.6 ± 0.0	–	–	0.08 ± 0.0	32.5

Abbreviations: IC_{50}, half-maximal inhibitory concentration; NC, not calculated; *SD*, standard deviation; SI, selectivity index.
[a]ClogP, octanol/water partition coefficient.
[b]IC_{50} on fibroblast cells NIH/3T3.
[c]IC_{50} on promastigote of *Leishmania amazonensis*.
[d]SI, IC_{50} on mammal cells/IC_{50} on promastigotes.
[e]CI_{50} on intracellular amastigote of *L. amazonensis*.
[f]SI, IC_{50} on mammal cells/IC_{50} on intracellular amastigotes.
[g]Positive control, pentamidine, and amphotericin B for *L. amazonensis* and doxorubicin for fibroblasts.

2.2.2 | Antifungal activity

Initially, the disk diffusion method was performed for determining the susceptibility of *C. parapsilosis* (ATCC 22019), *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 2001), and *C. tropicalis* (ATCC 750) to the nitrofuran isoxazole derivatives.

Nineteen nitrofuran isoxazole derivatives were tested, and compounds **14a**, **14d**, **14g**, **14h**, **14k**, **14m**, **14o**, **14p**, **14q**, and **14t** were active against one or more strains of *Candida* and were thus selected for the broth microdilution test.

All 3,5-nitrofuran isoxazole derivatives tested were active against *C. parapsilosis* (Table 4). Although this strain is less virulent than other *Candida* spp, this result is very interesting since *C. parapsilosis* is the most common candidemia-causing species in Latin America after *C. albicans*.[32]

These infections are related to the use of catheters and parenteral nutrition probably because of the capacity of colonizing plastic surfaces and forming of a biofilm. Moreover, an increase in the nonsusceptibility of *C. parapsilosis* to azole and amphotericin B has been reported.[33]

For the aromatic series, compound **14g** was found to be most active against *C. parapsilosis* with a minimal inhibitory concentration (MIC) of 5.7 μM, similar to fluconazole (MIC = 6.5 μM) used as the standard drug. Compound **14d** showed an MIC of 111 μM, whereas compound **14j** showed an MIC = 26.4 μM, which suggests an improvement in the biological activity due to the bioisosteric replacement of the oxygen atom to a sulfur atom (Table 4).

The alkylated compounds with the odd number of methylene groups **14k**, **14m**, and **14o** were active against *C. parapsilosis*. Compound **14o** has the highest ClogP of this series (Table 4), and it was found to be the most active with an MIC of 3.4 μM, lower than fluconazole and close to the amphotericin B, both used as positive controls for the test.

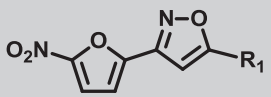
The chloroalkylated compounds **14p**, **14q**, and **14t** showed activity against two or more strains of *Candida*. In contrast to the alkylated compound series, the compound **14p** with the lowest ClogP showed an MIC of 8.7 μM. This derivative was active against *C. krusei* with MIC = 35 μM lower than the reference drug fluconazole (MIC = 52 μM) and also showed activity against all strains tested.

For *C. glabrata*, compound **14p** showed an MIC of 17.5 μM, lower than fluconazole. This is a promising result since *C. glabrata* has its mechanism of resistance related to azole compounds, such as fluconazole, due to the efflux pump.[34]

3 | CONCLUSION

Isoxazole nitrofuran series with structural diversity was synthesized with good yields. The antileishmanial activity showed that

TABLE 3 Antileishmanial activity of alkyl and chloroalkyl 3,5-disubstituted isoxazoles, **14k–r** and **14t**



14k–r and **14t**

Compound	ClogP[a]	NIH/3T3 IC_{50} (μM) ± *SD*[b]	Promastigote IC_{50} (μM) ± *SD*[c]	SI[d]	Intracellular amastigote IC_{50} (μM) ± *SD*[e]	SI[f]
14k: $R_1 = C_4H_9$	3.2	24.2 ± 1.6	211 ± 2.3	0.1	39.2 ± 1.6	0.6
14l: $R_1 = C_5H_{11}$	3.7	NC	200 ± 2.3	NC	19.4 ± 1.2	NC
14m: $R_1 = C_6H_{13}$	4.2	24.0 ± 1.5	16.4 ± 1.2	1.5	36.9 ± 1.6	0.6
14n: $R_1 = C_7H_{15}$	4.7	250 ± 2.3	3.3 ± 0.5	76.2	64.4 ± 1.8	3.9
14o: $R_1 = C_8H_{17}$	5.2	68.5 ± 5.8	6.1 ± 0.7	11.2	8.5 ± 0.9	8.0
14p: $R_1 = CH_2Cl$	2.1	3.1 ± 0.1	3.5 ± 0.5	0.8	0.6 ± 0.1	5.2
14t: $R_1 = (CH_2)_2Cl$	2.3	5.0 ± 2.3	206 ± 2.3	1.7	3.2 ± 0.4	1.6
14q: $R_1 = (CH_2)_3Cl$	2.6	7.0 ± 3.7	0.9 ± 0.0	8.0	3.8 ± 0.5	1.8
14r: $R_1 = (CH_2)_4Cl$	2.9	3.3 ± 0.4	1.8 ± 0.3	1.8	4.1 ± 0.6	0.8
Doxorubicin[g]	–	0.7 ± 0.0	–	–	–	–
Pentamidine[g]	–	27.5 ± 3.1	8.9 ± 0.6	3.0	3.3 ± 0.3	8.3
Amphotericin B[g]	–	2.6 ± 0.0	–	–	0.08 ± 0.0	32.5

Abbreviations: IC_{50}, half-maximal inhibitory concentration; NC, not calculated; *SD*, standard deviation; SI, selectivity index.
[a]ClogP, octanol/water partition coefficient.
[b]IC_{50} on fibroblast cells NIH/3T3.
[c]IC_{50} on promastigote of *Leishmania amazonensis*.
[d]SI, IC_{50} on mammal cells/IC_{50} on promastigotes.
[e]CI_{50} on intracellular amastigote of *L. amazonensis*.
[f]SI, IC_{50} on mammal cells/IC_{50} on intracellular amastigotes.
[g]Positive control, pentamidine and amphotericin B for *L. amazonensis* and doxorubicin for fibroblasts.

TABLE 4 Anti-*Candida* activity of 5-nitrofuran isoxazole derivatives

Compound	*Candida parapsilosis* MIC (μM ± *SD*)	*Candida albicans* MIC (μM ± *SD*)	*Candida krusei* MIC (μM ± *SD*)	*Candida tropicalis* MIC (μM ± *SD*)	*Candida glabrata* MIC (μM ± *SD*)
14a: R_1 = Ph	31.2 ± 1.5	–	–	–	–
14d: R_1 = 4-OCH_3–Ph	111.0 ± 2.0	–	–	–	–
14g: R_1 = 3,4,5-*tri*-OCH_3–Ph	5.7 ± 0.7	–	–	–	–
14h: R_1 = 3,4-methylenedioxy–Ph	53.2 ± 1.7	–	–	–	–
14j: R = SCH_3–Ph	26.4 ± 1.4	–	–	–	–
14k: $R_1 = C_4H_9$	135.5 ± 2.1	–	–	–	–
14m: $R_1 = C_6H_{13}$	7.6 ± 0.8	–	–	–	–
14o: $R_1 = C_8H_{17}$	3.4 ± 0.5	–	–	–	–
14p: $R_1 = CH_2Cl$	8.7 ± 0.9	35.0 ± 1.5	35.0 ± 1.5	35.0 ± 1.5	17.5 ± 1.2
14t: $R_1 = (CH_2)_2Cl$	66.0 ± 1.8	132.0 ± 2.1	132.0 ± 2.1	–	–
14q: $R_1 = (CH_2)_3Cl$	29.5 ± 1.5	118.0 ± 2.0	–	–	–
Fluconazole[a]	6.5 ± 0.8	0.8 ± 0.0	52.0 ± 1.7	3.3 ± 0.5	52.0 ± 1.7
Amphotericin B[a]	1.0 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	4.3 ± 0.6	8.6 ± 0.9

Abbreviations: MIC, minimal inhibitory concentration; *SD*, standard deviation.
[a]Fluconazole and amphotericin B used as positive controls.

the chloroalkyl-substituted compounds **14p–r** were active for both the promastigote and amastigote forms, emphasizing the activity of compound **14p** that showed IC_{50} = 0.6 μM and SI = 5.2 against the amastigote forms. In the alkyl series, compound **14o** stands out with an IC_{50} of 8.5 μM and SI of 8.0 for the amastigote forms. In the aromatic series, the most active compounds were those containing electron-donor groups such as trimethoxy isoxazole **14g** (IC_{50} = 1.2 μM and SI = 20.2), **14h** (IC_{50} = 7.0 μM

and SI = 6.1), and **14j** (IC_{50} = 5.7 μM and SI = 10.2). Thus, the compounds **14o**, **14p**, **14g**, **14h**, and **14j** are promising candidates for future in vivo studies, due to their relevant activity mainly on amastigotes, the target/aimed parasite form in this type of study. For the antifungal activity, eleven compounds were active against one or more strains of *Candida* in a broth microdilution test. All the compounds tested were active against *C. parapsilosis*, and compound **14o** was found to be the most active (MIC = 3.4 μM) to this strain. On the contrary, compound **14p** was active against all strains of *Candida* tested, with a minimal inhibitory concentration (MIC) for *C. krusei* and *C. glabrata* of 35.5 μM and 17.5 μM, respectively, lower than fluconazole.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General remarks

Anhydrous solvents were dried and distilled before use according to the standard procedure.[35] All chemicals used for synthesis were of reagent grade and used without purification unless noted otherwise. Reactions were carried out under a nitrogen atmosphere and monitored by thin-layer chromatography using prepared plates (silica gel 60 F254 on aluminum). The chromatograms were examined under both 254 and 360 nm ultraviolet light or with the developing agent ethanolic vanillin and heat. Flash-column chromatography was performed on silica gel 60 (particle size 200–400 mesh ASTM, purchased from Aldrich). Melting points were determined using Fisatom 430D equipment. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions using a Bruker 75- or 300-MHz spectrometer, as noted. Chemical shifts (δ) are expressed as parts per million (ppm) downfield from tetramethylsilane or deuterated solvent ($CDCl_3$ ^{1}H δ 7.27 and ^{13}C δ 77.0 ppm; dimethyl sulfoxide (DMSO)-d_6 ^{1}H δ 2.5 and ^{13}C δ 39.51 ppm; acetone ^{1}H δ 2.05 and ^{13}C δ 29.8 ppm) as the internal standard. HR-ESI-MS (high-resolution electrospray ionization mass spectrometry) measurements were carried out on a quadrupole time-of-flight instrument (UltrOTOF-Q; Bruker Daltonics, Billerica, MA).

Additional data are provided as Supporting Information. The InChI codes of the investigated compounds together with some biological activity data are also provided as Supporting Information.

4.1.2 | General procedure for the preparation of 3,5-nitrofuran isoxazole derivatives 14

To a solution of the respective chloro-oxime **17** (2 mmol, 1.0 equiv.) and the acetylene of interest **22a–s**, **25** (2.2 mmol, 1.1 equiv.) in CH_2Cl_2/THF (50:50, 6 ml), $CuSO_4.5H_2O$ (0.13 mmol) and sodium ascorbate (0.35 mmol) were added, and the mixture reaction was stirred for 10 min. Then, $KHCO_3$ (20 mmol, 10 equiv.) was added, and the mixture was stirred at room temperature for 48 hr. Extraction was performed with ethyl acetate (3 × 30 ml) and washed with a saturated solution of NH_4Cl (3 × 30 ml). The organic phase was dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The product was purified by recrystallization in heated ethanol.

*3-(5-Nitrofuran-2-yl)-5-phenylisoxazole (**14a**)*[36]

The product was obtained as a yellow solid in 89% yield. Mp: 182–184°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.97 (s, 1H), 7.18 (d, J = 3 Hz, 1H), 7.44 (d, J = 3 Hz, 1H), 7.49–7.51 (m, 3H), and 7.82–7.85 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 99.15, 114.76, 114.93, 126.27, 126.47, 129.86, 131.53, 146.05, 152.64, 154.14, and 171.06. HRMS (ESI+) m/z calcd for $C_{13}H_{18}N_2O_4$ [M+H]: 257.0562; found: 257.0578.

*5-(4-Chlorophenyl)-3-(5-nitrofuran-2-yl)isoxazole (**14b**)*[37]

The product was obtained as a yellow solid in 55% yield. Mp: 211–213°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.47 (d, J = 3.9 Hz, 1H), 7.62 (d, J = 8.5 Hz, 2H), 7.68 (s, 1H), 7.86 (d, J = 3.9 Hz, 1H), and 7.94 (d, J = 8.5 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): 99.69, 114.76, 114.96, 125.31, 128.10, 129.99, 136.19, 145.87, 152.66, 154.22, and 169.96. HRMS (ESI+) m/z calcd for $C_{13}H_7ClN_2O_4$ [M+H]: 291.0172; found: 291.0172.

*3-(5-Nitrofuran-2-yl)-5-(4-nitrophenyl)isoxazole (**14c**)*

The product was obtained as a yellow solid in 45% yield. Mp: 230–232°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.57 (d, J = 3 Hz, 1 H), 7.91 (d, J = 3 Hz, 1H), 7.98 (s, 1H), 8.24 (d, J = 9 Hz, 2H), 8.42 (d, J = 9 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 101.58, 114.34, 114.89, 124.70, 127.23, 131.47, 145.14, 148.55, 154.07, 168.48. HRMS (ESI+) m/z calcd for $C_{13}H_7N_3O_6$ [M+H]: 302.0413; found: 302.0469.

*5-(4-Methoxyphenyl)-3-(5-nitrofuran-2-yl)isoxazole (**14d**)*

The product was obtained as a brown solid in 83% yield. Mp: 164–166°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.81 (s, 3H), 7.09 (d, J = 9 Hz, 2H), 7.45–7.47 (m, 2H), and 7.85–7.87 (m, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 55.90, 97.54, 114.69, 114.76, 115.25, 119.12, 128.04, 146.26, 152.58, 154.02, 161.75, and 171.13. HRMS (ESI+) m/z calcd for $C_{14}H_{10}N_2O_5$ [M+H]: 287.0668; found: 287.0664.

*5-(3,4-Dimethoxyphenyl)-3-(5-nitrofuran-2-yl)isoxazole (**14e**)*

The product was obtained as a yellow solid in 85% yield. Mp: 240–242°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.80 (s, 6H), 6.64 (t, J = 2.1 Hz, 1H), 7.07 (d, J = 2.1 Hz, 2H), 7.45 (d, J = 3.9 Hz, 1H), 7.69 (s, 1H), and 7.86 (d, J = 3.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 56.07, 99.71, 103.60, 104.13, 114.78, 128.10, 146.02, 152.65, 154.15, 161.51, 170.92. HRMS (ESI+) m/z calcd for $C_{15}H_{12}N_2O_6$ [M+H]: 317.0773; found: 317.0765.

*5-(3,5-Dimethoxyphenyl)-3-(5-nitrofuran-2-yl)isoxazole (**14f**)*

The product was obtained as a yellow solid in 72% yield. Mp: 216–218°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.80 (s, 6H), 6.65 (s, 1H), 7.07 (d, J = 3.9 Hz, 2H), 7.45 (d, J = 3 Hz, 1H), 7.69 (s, 1H), and 7.85 (d, J = 3.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 56.08, 99.71,

103.62, 104.17, 114.77, 128.12, 146.04, 152.15, 154.15, 161.53, and 170.94. HRMS (ESI+) *m/z* calcd for $C_{15}H_{12}N_2O_6$ [M+H]: 317.0773; found: 317.0780.

*3-(5-Nitrofuran-2-yl)-5-(3,4,5-trimethoxyphenyl)isoxazole (**14g**)*

The product was obtained as a yellow solid in 81% yield. Mp: 189–191°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.90 (s, 3H), 3.94 (s, 6H), 6.92 (s, 1H), 7.04 (s, 2H), 7.18 (d, J = 3 Hz, 1H), and 7.44 (d, J = 3 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.35, 61.04, 96.90, 103.31, 111.95, 113.09, 121.75, 140.49, 146.60, 153.80, 153.89, and 171.39. HRMS (ESI+) *m/z* calcd for $C_{16}H_{14}N_2O_7$ [M+H]: 347.0879; found: 347.0884.

*5-(Benzo[d][1,3]dioxol-5-yl)-3-(5-nitrofuran-2-yl)isoxazole (**14h**)*

The product was obtained as as brown solid in 74% yield. Mp: 162–164°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.11 (s, 2H), 7.08 (d, J = 6 Hz, 1H), 7.43–7.49 (m, 4H), and 7.85 (d, J = 3 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 98.06, 102.38, 106.42, 109.56, 114.66, 114.77, 120.47, 121.17, 146.16, 148.62, 150.04, 152.62, 154.06, and 170.93. HRMS (ESI+) *m/z* calcd for $C_{14}H_8N_2O_6$ [M+H]: 301.0460; found: 301.0456.

*3-(5-Nitrofuran-2-yl)-5-(4-phenoxyphenyl)isoxazole (**14i**)*

The product was obtained as a yellow solid in 64% yield. Mp: 183–185°C. ^{1}H NMR (300 MHz, acetone-d_6) δ 7.09–7.14 (m, 4H), 7.19–7.24 (t, J = 7.4 Hz, 1H), 7.32 (s, 1H), 7.37 (d, J = 3.8 Hz, 1H), 7.41–7.47 (m, 2H), 7.68 (d, J = 3.8 Hz, 1H), and 7.97 (d, J = 8.9 Hz, 2H). ^{13}C NMR (75 MHz, acetone-d_6) δ 97.1, 113.1, 133.3, 118.4, 119.8, 121.4, 124.4, 127.9, 130.2, 146.3, 154.0, 155.9, 160.0, and 170.7. HRMS (ESI+) *m/z* calcd for $C_{19}H_{12}N_2O_5$ [M+H]: 349.0824; found: 349.0814.

*5-(4-(Methylthio)phenyl)-3-(5-nitrofuran-2-yl)isoxazole (**14j**)*

The product was obtained as a brown solid with 58% yield. Mp: 138–140°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.51 (s, 3H), 7.38 (m, 4H), 7.50 (d, J = 9 Hz, 2H) and 7.88 (d, J = 3 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.50, 108.52, 114.69, 114.92, 124.31, 125.62, 126.64, 130.13, 142.95, 147.02, 152.59, 157.49, and 176.90. HRMS (ESI+) *m/z* calcd for $C_{19}H_{12}N_2O_5$ [M+H]: 303.0440; found: 303.0433.

*5-Butyl-3-(5-nitrofuran-2-yl)isoxazole (**14k**)*

The product was obtained as a brown solid in 86% yield. Mp: 88–90°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.91 (t, J = 6 Hz, 3H), 1.33 (q, J = 6 Hz, 2H), 1.68 (qt, J = 6 Hz, 2H), 2.84 (t, J = 6 Hz, 2H), 6.90 (s, 1H), 7.48 (d, J = 3 Hz, 1H), and 7.86 (d, J = 3 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.94, 21.93, 25.90, 29.30, 100.17, 114.77, 114.90, 146.42, 152.46, 153.28, and 175.72. HRMS (ESI+) *m/z* calcd for $C_{11}H_{12}N_2O_4$ [M+H]: 237.0875; found: 237.0872.

*3-(5-Nitrofuran-2-yl)-5-pentylisoxazole (**14l**)*

The product was obtained as a yellow solid in 70% yield. Mp: 91–93°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.83–0.85 (m, 3H), 1.27–1.30 (m, 4H), 1.64 (t, J = 6 Hz, 2H), 2.79 (t, J = 6 Hz, 2H), 6.85 (s, 1H), 7.42 (d, J = 3 Hz, 1H), and 7.81 (d, J = 3 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.21, 22.14, 26.19, 26.93, 30.99, 100.05, 114.66, 114.69, 146.45, 152.52, 153.28, and 175.76. HRMS (ESI+) *m/z* calcd for $C_{12}H_{14}N_2O_4$ [M+H]: 251.1032; found: 251.1058.

*5-Hexyl-3-(5-nitrofuran-2-yl)isoxazole (**14m**)*

The product was obtained as a brown solid in 91% yield. Mp: 92–94°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.87–0.91 (t, J = 6 Hz, 3H), 1.35–1.36 (m, 6H), 1.69–1.77 (qt, J = 6 Hz, 2H), 2.81 (t, J = 6 Hz, 2H), 6.45 (s, 1H), 7.11 (d, J = 3 Hz, 1H), 7.42 (d, J = 3 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.32, 22.35, 26.21, 27.19, 28.44, 31.21, 100.14, 114.75, 114.85, 146.42, 152.46, 153.26, and 175.71. HRMS (ESI+) *m/z* calcd for $C_{13}H_{16}N_2O_4$ [M+H]: 265.1188; found: 265.1192.

*5-Heptyl-3-(5-nitrofuran-2-yl)isoxazole (**14n**)*

The product was obtained as a yellow solid in 89% yield. Mp: 95–97°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.81 (m, 3H); 1.21–1.25 (m, 8H), 1.62 (t, J = 6 Hz, 2H), 2.78 (t, J = 6 Hz, 2H), 6.83 (s, 1H), 7.42 (d, J = 3.2 Hz, 1H), and 7.81 (d, J = 3.2 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.33, 22.47, 26.23, 27.26, 28.69, 28.77, 31.53, 100.03, 114.63, 114.68, 146.47, 152.50, 153.27, and 175.75. HRMS (ESI+) *m/z* calcd for $C_{14}H_{18}N_2O_4$ [M+H]: 279.1345; found: 279.1347

*3-(5-Nitrofuran-2-yl)-5-octylisoxazole (**14o**)*

The product was obtained as a yellow solid in 84% yield. Mp: 97–99°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.78–0.8 (m, 3H), 1.20–1.31 (m, 10H), 1.60-1.65 (m, 2H), 2.78 (t, J = 7.4 Hz, 2H), 6.84 (s, 1H), 7.43 (d, J = 3.9 Hz, 1H), 7.82 (d, J = 3.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.37, 22.51, 26.23, 27.25, 28.82, 28.98, 29.00, 31.67, 100.04, 114.67, 114.71, 146.45, 152.50, 153.27, 175.75. HRMS (ESI+) *m/z* calcd for $C_{15}H_{20}N_2O_4$ [M+H]: 293.1501; found: 293.1512.

*5-(Chloromethyl)-3-(5-nitrofuran-2-yl)isoxazole (**14p**)*

The product was obtained as a yellow solid in 68% yield. Mp: 87–89°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.02 (s, 2H), 7.18 (s, 1H), 7.52 (d, J = 3.9 Hz, 1H), and 7.83 (d, J = 3.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 34.49, 103.07, 114.60, 115.46, 145.53, 152.68, 153.66, and 169.95. HRMS (ESI+) *m/z* calcd for $C_8H_5ClN_2O_4$ [M+H]: 229.0016; found: 229.0012.

*5-(3-Chloropropyl)-3-(5-nitrofuran-2-yl)isoxazole (**14q**)*

The product was obtained as yellow solid in 74% yield. Mp: 94–96°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.11 (qt, J = 6 Hz, 2H), 2.96 (t, J = 6 Hz, 2H), 3.68 (t, J = 6 Hz, 2H), 6.90 (s, 1H), 7.42 (d, J = 3.7 Hz, 1H), and 7.80 (d, J = 3.7 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 23.81, 30.12, 44.67, 100.45, 114.66, 114.71, 146.32, 152.51, 153.36, and 174.32. HRMS (ESI+) *m/z* calcd for $C_{10}H_9ClN_2O_4$ [M+H]: 257.0329; found: 257.0342.

*5-(4-Chlorobutyl)-3-(5-nitrofuran-2-yl)isoxazole (**14r**)*

The product was obtained as a yellow solid in 78% yield. Mp: 96–98°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.74–1.79 (m, 4H),

2.84–2.88 (m, 2H), 3.63–3.68 (m, 2H), 6.88 (s, 1H), 7.44 (d, J = 3.6 Hz, 1H), and 7.82 (d, J = 3.6 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 29.34, 30.21, 36.44, 50.05, 104.98, 119.47, 119.51, 151.11, 151.27, 158.09, and 180.08. HRMS (ESI+) m/z calcd for $C_{11}H_{11}ClN_2O_4$ [M+H]: 271.0485; found: 271.0482.

*2-(3-(5-Nitrofuran-2-yl)isoxazol-5-yl)ethanol (**14s**)*

The product was obtained as a yellow solid in 30%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.95 (t, J = 6 Hz, 2H), 3.71 (q, J = 6 Hz, 2H), 4.91 (t, J = 6 Hz, 1H), 6.86 (s, 1H), 7.44 (d, J = 3 Hz, 1H), and 7.81 (d, J = 3 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 30.39, 58.78, 100.75, 114.65, 114.72, 146.46, 152.52; 153.28, and 174.00. HRMS (ESI+) m/z calcd for $C_9H_8N_2O_5$ [M+H]: 225.0511; found: 225.0507.

*5-(2-Chloroethyl)-3-(5-nitrofuran-2-yl)isoxazole (**14t**)*

To a solution of the respective of 2,4,6-trichloro-(1,3,5)-triazine (2 mmol, 1.05 eq.) in dimethylformamide (1 ml) under magnetic stirring until total consumption of the starting material (approximately 10 min), then the synthesized isoxazole **14s** (2 mmol, 1.0 eq.) dissolved in CH_2Cl_2 (5 ml) was added dropwise. This reaction mixture was stirred at room temperature for 4 hr. Then, 30 ml of saturated NaCl solution was added and the product was extracted with ethyl acetate (2 × 30 ml). The organic phase was dried with anhydrous $MgSO_4$ and the solvent evaporated under reduced pressure. The product was purified by crystallization in heated ethanol. The product was obtained as a yellow solid in 65% yield. Mp: 91–93ºC. ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.33 (m, 2H), 3.96 (t, J = 6 Hz, 2H), 7.00 (s, 1H), 7.47 (d, J = 3.9 Hz, 1H), and 7.83 (d, J = 3.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 29.8, 42.02, 101.43, 114.71, 114.85, 146.11, 152.59, 153.43, and 172.30. HRMS (ESI+) m/z calcd for $C_9H_7ClN_2O_4$ [M+H]: 243.0172; found: 243.0138.

4.2 | Biological methods

4.2.1 | Antileishmanial assays[11–14,38–40]

L. (L.) amazonensis promastigotes

In 96-well microplates, the synthetic compounds **14a–t** were tested in quintuplicates, with concentrations from 0.78 μg/ml to 50 μg/ml. Promastigotes forms of *L. amazonensis* (IFLA/BR/1967/PH8 strain) cultivated in Schneider's Insect Medium (Sigma-Aldrich®, St. Louis, MO) were added to the wells in the exponential phase with a concentration of 2 × 10^5 parasites/ml. The plates were incubated at 26°C for 72 hr. To evaluate the cell viability, 20 μl (5 mg/ml) of Thiazolyl Blue Tetrazolium Bromide—MTT (Sigma-Aldrich®) was added in each well and incubated at 37°C, 5% CO_2 for 2 hr. After that, 80 μl of DMSO was added to finish the reaction.

Pentamidine (Sigma-Aldrich®) was used as a positive control. DMSO (Sigma-Aldrich®), in Schneider's Insect Medium, was used as negative control and did not interfere with cell viability. The reaction results were obtained in a spectrophotometer (Biotek®) at 540 nm. A nonlinear dose–response regression curve was used to calculate the half-maximum inhibitory concentration (IC_{50}).

The percentage of activity was calculated by the following formula:

$$\% \text{ Activity} = 100 - [(N - Y)/(N - P)] \times 100,$$

where Y is the optical density reading of cells and wells with different concentrations of the compounds, N is the optical density reading of parasites in wells with 1.5% DMSO, and P is the optical density reading of the wells with only culture medium.

Pentamidine (IC_{50}) promastigotes = 8.9 μM.

Parasites and peritoneal macrophages—amastigotes anti-Leishmania evaluation[11–14,38–40]

In vitro antileishmanial activity was evaluated on peritoneal macrophages infected with. *L.* (*L.*) *amazonensis* intracellular amastigotes. Parasites (IFLA/BR/1967/PH8 strain) were routinely isolated from BALB/c mice and maintained as promastigotes at 25°C in Schneider's Insect Medium (Sigma-Aldrich®) supplemented with 20% fetal calf serum (FCS-Cultilab® Campinas, Brazil) and 140 μg/ml gentamicin (Sigma-Aldrich®). Macrophages were obtained from the peritoneal wash of BALB/c mice after euthanasia. 10 ml of RPMI-1640 (Sigma-Aldrich®) supplemented with 2% L-glutamine, 2.8% bicarbonate buffer, 100 U/ml penicillin and 100 mg/ml streptomycin were injected into the peritoneal cavity. After the massage area, the liquid was aspirated and transferred to tubes on ice. Peritoneal cells were quantified in a Neubauer chamber after cellular exclusion with Trypan Blue staining (Sigma-Aldrich®).

In vitro antileishmanial activity on intracellular amastigotes[11–14,38–40]

Peritoneal cells (1 × 10^5 cells/well) were added to 24-well plates containing circular coverslips. The plates were incubated for 1 hr at 37°C/5% CO_2 to allow cell adhesion and then 1 × 10^6 *L. (L.) amazonensis* promastigotes were added to each well. Plates were incubated at 35°C/5% CO_2 for 4 hr and then cells were treated for 24 hr with synthetic compounds **14a–t** (6.25–50 μg/ml). Amphotericin B (Sigma-Aldrich®) was used as the reference drug (0.25 to 2 μg/ml) and untreated cells were used as a negative control. Coverslips were processed as described by Rizk et al.[39] The overall number of amastigotes was determined by counting 100 cells in six replicates. The half-maximal inhibitory concentration (IC_{50}) was calculated using a nonlinear regression curve. The infection index was obtained as described by Paladi et al.[40]

4.2.2 | Cytotoxicity assay[11–14,38–40]

Fibroblasts (NIH/3T3) obtained from Rio de Janeiro Cell Bank (Brazil) were seeded in 96-well plates (1 × 10^4 cells/ml) and incubated with synthetic compounds at 37°C, 5% CO_2 for 48 hr at the concentration of 0.25–250 μg/ml.[13] Doxorubicin (Sigma-Aldrich®) was used as the reference drug at concentrations of 0.025–25 μg/ml. Cell growth was estimated by the sulforhodamine B colorimetric method.[41] DMSO (Vetec) was used as a

negative control at a concentration necessary to solubilize the highest concentration of the test sample and did not interfere with cell viability. The percentage of growth was calculated as described by Monks et al.[42] IC_{50} was determined by nonlinear regression analysis (Microcal Origin Versão 6.0®) and Microsoft Office Excell 2007®. SI was calculated according to Medeiros et al.[38]

4.2.3 | Antifungal assays

Disk diffusion method

The strains of C. parapsilosis (ATCC 22019), *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 2001), and *C. tropicalis* (ATCC 750) were used for the disk diffusion test.

The procedure was performed according to the protocol M44-A2 of the Clinical Laboratory Standards Institute (CLSI, 2009).[43]

The yeast was subcultured onto Sabouraud-dextrose agar and incubated at 35°C (±2°C) for 24 hr. The inoculums of yeast strains were prepared by selecting colonies from a 24-hr-old culture of *Candida* spp and suspended in 5 ml of sterile 0.145 mol/l saline (0.85% saline). Its turbidity was adjusted with a spectrometer according to 0.5 McFarland standard resulting in a stock suspension of 1×10^6 to 5×10^6 cells/ml. Each yeast strain was inoculated with a sterile swab into Mueller–Hinton + GMB agar plates. A solution of each nitrofuran isoxazole derivative at concentrations of 5 and 2.5 mg/ml was prepared and added to the paper discs placed onto the inoculated plate. The plates were incubated at 35°C (±2°C) for 24 hr, and the fungal susceptibility was determined by measuring the diameter of the inhibition zone. Assays were performed in duplicate, and DMSO was used as the negative control, whereas fluconazole and amphotericin B were used as positive controls.

Determination of minimal inhibitory concentration

The MIC was determined only for the active compounds that showed a growth inhibition zone on the disk diffusion test. The procedure was performed according to the protocol M27-A3 of the Clinical Laboratory Standards Institute.[44]

The yeast was subcultured onto Sabouraud-dextrose agar and incubated at 35°C (±2°C) for 24 hr. The inoculums of yeast strains were prepared by selecting colonies from a 24-hr-old culture of Candida spp and suspended in 5 ml of sterile 0.145 mol/l saline (0.85% saline). Its turbidity was adjusted with a spectrometer according to 0.5 McFarland standard, resulting in a stock suspension of 1×10^6 to 5×10^6 cells/ml. A working suspension was firstly diluted 1:50 and then 1:20 in Roswell Park Memorial Institute (RPMI)-1640 broth medium without antibiotic and bicarbonate, which results in 1×10^3–5×10^3 UFC/ml.

Initially, 51.2 μl of 5 mg/ml solution of 3,5-nitrofuran compound was added to 1,948.8 μl of RPMI-1640 to give 128 μg/ml of concentration.

In columns 2 to 11 of a 96-well microplate were added RPMI-1640 (100 μl) and solution of 3,5-nitrofuran isoxazole (100 μl). Serial dilution was carried out, resulting in the final concentration of 64 μg/ml in row 2 and 0.125 μg/ml in row 11 followed by the addition of inoculum suspension (100 μl). RPMI-1640 (200 μl) was added to column 1 and used as the negative control. RPMI-1640 (100 μl) and the inoculum (100 μl) were added to the column 12 and used as the positive control. Fluconazole was used at a concentration of 64 μg/ml to 0.125 μg/ml. Amphotericin B was used at a concentration of 16–0.03 μg/ml. The plates were sealed and incubated at 35°C (±2°C) for 24 hr. The tests were performed in triplicate. The result was observed in a spectrophotometer (Spectramax 190 microplate reader) at 530 nm. The MIC was defined as the lowest concentration that causes a reduction in the visible growth of a microorganism when compared with the positive control.

ACKNOWLEDGMENTS

We thank Dr. Janet W. Reid (JWR Associates) for her assistance with English corrections. Special thanks to the Laboratory of Natural Products and Mass Spectrometry (LaPNEM) of the Federal University of Mato Grosso do Sul for the HPLC-DAD-MS/MS analysis. This study was supported by grants from FUNDECT-MS (Process numbers 59/300.109/2015, 23/200.462/2014, 23/200.708/2013), CNPq, and CAPES.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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REFERENCES

[1] World Health Organization (WHO), *Wkly. Epidemiol. Rec.* **2016**, *22*, 287.
[2] L. F. Oliveira, A. O. Schubach, M. M. Martins, S. L. Passos, R. V. Oliveira, M. C. Marzochi, C. A. Andrade, *Acta Trop.* **2011**, *118*, 87.
[3] J. N., Sangshetti, F. A. K. Khan, A. A. Kulkarni, R. Aroteb, R. H. Patilc, *RSC Adv.* **2015**, *5*, 32376.
[4] J. A. L. Lindoso, J. M. L. Costa, I. T. Queiroz, H. Goto, *J. Res. Rep. Trop. Med.* **2012**, *3*, 69.
[5] P. G. Pappas, M. S. Lionakis, M. C. Arendrup, L. Ostrosky-Zeichner, B. J. Kullberg, *Nat. Rev.* **2018**, *4*, 1.
[6] N. Thamban chandrika, S. K. Shrestha, H. X. Ngo, K. C. Howard, S. Garneau-Tsodikova, *Bioorg. Med. Chem.* **2018**, *26*, 573.
[7] F. Lamoth, S. R. Lockhart, E. L. Berkow, T. Calandra, *Antimicrob. Chemother.* **2018**, *73*, i4.
[8] S. Campoy, J. L. Adrio, *Biochem. Pharmacol.* **2017**, *133*, 86.
[9] D. W. Denning, M. J. Bromley, *Science* **2015**, *27*, 1414.
[10] A. A. Da Silva Filho, E. S. Costa, W. R. Cunha, M. L. A. Silva, N. P. D. Nanayakkara, J. K. Bastos, J. K. Bastos, *Phytother. Res.* **2008**, *22*, 1307.

[11] O. S. Trefzger, A. R. Neves, N. V. Barbosa, D. B. Carvalho, I. C. Pereira, R. T. Perdomo, M. F. C. Matos, N. C. Yoshida, M. J. Kato, S. Albuquerque, C. C. P. Arruda, A. C. M. Baroni, *Chem. Biol. Drug. Des.* **2019**, *93*, 313.
[12] A. R. Neves, O. S. Trefzger, N. V. Barbosa, A. M. Honorato, D. B. Carvalho, I. S. Moslaves, M. C. T. Kadri, N. C. Yoshida, M. J. Kato, C. C. P. Arruda, A. C. M. Baroni, *Chem. Biol. Drug. Des.* **2019**, *94*, 2004. https://doi.org/10.1111/cbdd.13609
[13] T. B. Cassamale, E. C. Costa, D. B. Carvalho, N. S. Cassemiro, C. C. Tomazela, M. C. S. Marques, M. Ojeda, M. F. C. Matos, S. Albuquerque, C. C. P. Arruda, A. C. M. Baroni, *J. Braz. Chem. Soc.* **2016**, *27*, 1217.
[14] E. Costa, T. Cassamale, D. Carvalho, L. Bosquiroli, M. Ojeda, T. Ximenes, M. Matos, M. Kadri, A. Baroni, C. Arruda, *Molecules* **2016**, *21*, 802.
[15] K. J. M. Swapnaja, S. Yennam, M. Chavali, Y. Poornachandra, C. G. Kumar, K. Muthusamy, V. B. Jayaraman, P. Arumugan, S. Balasubramanian, K. K. Sriram, *Eur. J. Med. Chem.* **2016**, *117*, 8588.
[16] X. Liang, F. Lv, B. Wang, K. Yu, *J. Med. Chem.* **2017**, *60*, 1580.
[17] S. R. Pedada, N. S. Yarla, P. J. Tambade, B. L. Dhananjaya, A. Bishayee, K. M. Arunasree, G. H. Philip, G. Dharmapuri, G. Aliev, S. Putta, G. Rangaiah, *Eur. J. Med. Chem.* **2016**, *112*, 289.
[18] J. Zhu, J. Mo, H. Lin, Y. Chen, H. Sun, *Biorg. Med. Chem.* **2018**, *26*, 3065.
[19] R. Da Rosa, M. H. de Moraes, L. A. Zimmermann, E. P. Schenkel, M. Steindel, L. S. C. Bernardes, *Eur. J. Med. Chem.* **2017**, *128*, 25.
[20] M. Tomishima, H. Ohki, A. Yamada, K. Maki, F. Ikeda, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2886.
[21] F. Chevreuil, A. Landreau, D. Séraphin, G. Larcher, S. Mallet, J.-P. Bouchara, P. Richomme, *J. Enzyme Inhib. Med. Chem.* **2007**, *22*, 563.
[22] M. C. Chung, R. V. C. Güido, T. F. Martinelli, M. F. Gonçalves, M. C. Polli, K. C. A. Botelho, E. A. Varanda, W. Colli, M. T. M. Miranda, E. I. Ferreira, *Bioog. Med. Chem.* **2003**, *11*, 4779.
[23] A. Kamal, S. M. A. Hussaini, M. L. Sucharitha, Y. Poornachandra, F. Sultana, C. Ganesh Kumar, *Org. Biomol. Chem.* **2015**, *13*, 9388.
[24] M. Chin Chung, P. Longhin Bosquesi, J. Leandro dos Santos, *Curr. Pharm. Des.* **2011**, *17*, 3515.
[25] S. Wyllie, S. Patterson, L. Stojanovski, F. R. C. Simeons, S. Norval, R. Kime, K. D. Read, A. H. Fairlamb, *Sci. Transl. Med.* **2012**, *4*, 119re1.
[26] V. K. B. K. Mesu, W. M. Kalonji, C. Bardonneau, O. V. Mordt, S. Blesson, F. Simon, S. Delhomme, S. Bernhard, W. Kuziena, J.-P. F. Lubaki, S. L. Vuvu, P. N. Ngima, H. M. Mbembo, M. Ilunga, A. K. Bonama, J. A. Heradi, J. L. L. Solomo, G. Mandula, L. K. Badibabi, F. R. Dama, P. K. Lukula, D. N. Tete, C. Lumbala, B. Scherrer, N. Strub-Wourgaft, A. Tarral, *Lancet* **2018**, *391*, 144.
[27] D. G. Rando, M. A. Avery, B. L. Tekwani, S. I. Khan, E. I. Ferreira, *Bioorg. Med. Chem.* **2008**, *16*, 6724.
[28] S. B. Bharate, A. K. Padala, B. A. Dar, R. R. Yadav, B. Singh, R. A. Vishwakarma, *Tetrahedron Lett.* **2013**, *54*, 3558.
[29] A. A. Vieira, F. R. Bryk, G. Conte, A. J. Bortoluzzi, H. Gallardo, *Tetrahedron Lett.* **2009**, *50*, 905.
[30] L. de Luca, G. Giacomelli, A. Porcheddu, *Org. Lett.* **2002**, *4*, 553.
[31] C. G. Wermuth, *The Practice of Medicinal Chemistry*, 3rd ed., Academic Press, San Diego, CA **2008**.
[32] D. A. Matta, A. C. R. Souza, A. L. Colombo, *J. Fungi* **2017**, *27*, 24.
[33] L. S. Ruiz, S. Khouri, R. C. Hahn, E. G. Da Silva, V. K. P. de Oliveira, R. F. Gandra, C. R. Paula, *Mycophatologia* **2013**, *175*, 231.
[34] S. Brun, T. Berges, P. Poupard, C. Vauzelle-Moreau, G. Renier, D. Chabasse, J.-P. Bouchara, *Antimicrob. Agents Chemother.* **2004**, *48*, 1788.
[35] W. L. F. Amarego, D. D. Perrin, *Purification of Laboratory Chemicals*, 3rd ed., Pergamon Press, New York, NY **1988**.
[36] S. B. Bharate, A. K. Padala, B. A. Dar, R. R. Yadav, B. Singh, R. A. Vishwakarma, *Tetrahedron Lett.* **2013**, *54*, 3558.
[37] R. G. Haber, E. Schoenberger, *Israel J. Chem.* **1968**, *6*, 631.
[38] M. G. F. de Medeiros, A. C. Da Silva, A. M. G. L. Citó, A. R. Borges, S. G. de Lima, J. A. D. Lopes, *Parasitol. Int.* **2011**, *60*, 237.
[39] Y. S. Rizk, A. Fischer, M. C. Cunha, P. O. Rodrigues, M. C. S. Marques, M. F. C. Matos, M. C. T. Kadri, C. A. Carollo, C. C. P. Arruda, *Mem. Inst. Oswaldo Cruz* **2014**, *109*, 1050.
[40] C. S. Paladi, I. A. S. Pimentel, S. Katz, R. L. O. R. Cunha, W. A. S. Judice, A. C. F. Caires, C. L. Barbiéri, C. L. Barbiéri, *PLOS Neglect. Trop. Dis.* **2012**, *6*, e1626.
[41] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, M. R. Boyd, *J. Natl. Cancer Inst.* **1990**, *82*, 1107.
[42] A. Monks, D. Scudiero, P. Skehan, R. Shoemaker, K. Paull, D. Vistica, C. Hose, J. Langley, P. Cronise, A. Vaigro-Wolff, M. Gray-Goodrich, H. Campbell, J. Mayo, M. Boyd, *J. Natl. Cancer Inst.* **1991**, *83*, 757.
[43] Clinical and Laboratory Standards Institute (CLSI). *Method for antifungal disk diffusion susceptibility testing of yeasts*. CLSI Document M44-A2, Wayne, PA, **2009**, 29, 1-25.
[44] Clinical and Laboratory Standards Institute. *Reference method for broth dilution antifungal susceptibility testing of yeasts*. CLSI Document M27-A3. Wayne, PA, **2008**, 28, 1-25.

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How to cite this article: Trefzger OS, Barbosa NV, Scapolatempo RL, et al. Design, synthesis, antileishmanial, and antifungal biological evaluation of novel 3,5-disubstituted isoxazole compounds based on 5-nitrofuran scaffolds. *Arch Pharm Chem Life Sci*. 2019;e1900241. https://doi.org/10.1002/ardp.201900241